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Identification of the Murine Cytomegalovirus Glycoprotein B Gene and Its Expression by Recombinant Vaccinia Virus

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The gene encoding glycoprotein B (gB) of murine cytomegalovirus (MCMV) strain Smith was identified, sequenced, and expressed by recombinant vaccinia virus. The gB gene was found adjacent to the polymerase gene, as it is in the genome of human cytomegalovirus (HCMV). The open reading frame consists of 2,784 nucleotides capable of encoding a protein of 928 amino acids. Comparison with gB homologs of other herpesviruses revealed a high degree of homology. The similarity between the MCMV gB and the HCMV gB is most prominent, since 45% of the amino acids are identical. In addition, all cysteine residues are at homologous positions, indicating a similar tertiary structure of the two proteins. In contrast to HCMV, the MCMV gB mRNA is a true late transcript. A recombinant vaccinia virus expressing the MCMV gB gene has been constructed (Vac-gB). Antibodies raised against the Vac-gB recombinant precipitated proteins of 130, 105, and 52 kDa from MCMV-infected cells. The identity of the MCMV gB with the major envelope glycoprotein of MCMV described by Loh et al. was shown (L. C. Loh, N. Balachandran, and L. F. Qualtiere, Virology 166:206–216, 1988). Immunization of mice with the Vac-gB recombinant gave rise to neutralizing antibodies.

Murine cytomegalovirus (MCMV) and human cytomegalovirus (HCMV) are members of the subfamily *Betaherpesvirinae*. HCMV is a ubiquitous human pathogen, but most primary infections do not lead to an apparent disease. However, HCMV is a major cause of serious illness in congenitally infected infants and in immunocompromised individuals (15, 34). Patients with AIDS, organ allograft recipients, and cancer patients receiving immunosuppressive drugs develop severe and often fatal HCMV infections. At present only live, attenuated virus strains which still bear the risk of latent infections are available as vaccines. The severity of the CMV infection may be moderated by the administration of anti-CMV antibodies (37). The efficacy of the vaccines and the value of the antibody treatment are controversial (41). A better characterization and understanding of the host immune response against CMV would facilitate the search for new and improved vaccines and targeted treatments.

Envelope glycoproteins of herpesviruses represent dominant antigens for both the cellular and the humoral immune response (40). Their property of serving as targets for neutralizing antibodies is of particular interest. The major envelope glycoprotein complex of HCMV is called gC-I (16). The glycoprotein complex gC-I consists of two mature glycoproteins with molecular masses of 93 to 130 kDa and 52 to 58 kDa, derived by proteolytic cleavage from a precursor glycoprotein of 150 to 170 kDa (4, 9, 38). The proteins are encoded by a gene referred to as gB by Cranage et al. (9) and Mach et al. (30) because of its sequence homology with the glycoprotein B (gB) gene of herpes simplex virus (HSV). A significant fraction of the neutralizing antibodies in human antisera are directed against the proteins of the gC-I complex (4). In addition, antisera raised against vaccinia virus recombinants expressing gB or monoclonal anti-gB antibodies effectively neutralize the virus in vitro (9). These data suggest that the glycoprotein B is an important target for the humoral immune response against HCMV.

Due to the species specificity of HCMV, in vivo experiments cannot be conducted. Therefore, the availability of an appropriate animal model is important. MCMV has been useful for studying CMV infections because of the many similarities between MCMV and HCMV in biology and pathogenesis. Thus, the study of the murine immune response to MCMV may be valuable for understanding the situation in humans.

In this report, we describe the identification and sequencing of a gene whose primary translation product shows characteristics of a glycoprotein. Comparison of the deduced amino acid sequence of the protein with sequences of other herpesvirus glycoproteins reveals extensive homology to the glycoprotein B of HCMV. The gB gene was expressed by recombinant vaccinia virus. The Vac-gB recombinant induced neutralizing antibodies in mice and thus provides a tool to test the usefulness of a gB subunit vaccine in an animal model.

MATERIALS AND METHODS

Virus and cell culture. MCMV (mouse salivary gland virus strain Smith [ATCC VR-194]) was propagated on BALB/c mouse embryonal fibroblasts as described previously (18). CV-1 cells, used for the propagation of vaccinia viruses, were maintained in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, 100 μg of streptomycin per ml, and 100 U of penicillin per ml. Mouse embryonal fibroblasts were MCMV infected with 20 PFU per cell by using the technique of centrifugal enhancement of infectivity at 800 × *g* for 30 min. Cell proteins were labeled with 125 μCi of [^{35}S]methionine (Amersham, Braunschweig, Germany) per ml of methionine-free medium.

Sequence analysis. The 5′-terminal part of the gB nucleic acid sequence (nucleotides [nt] −20 to 1400) was determined

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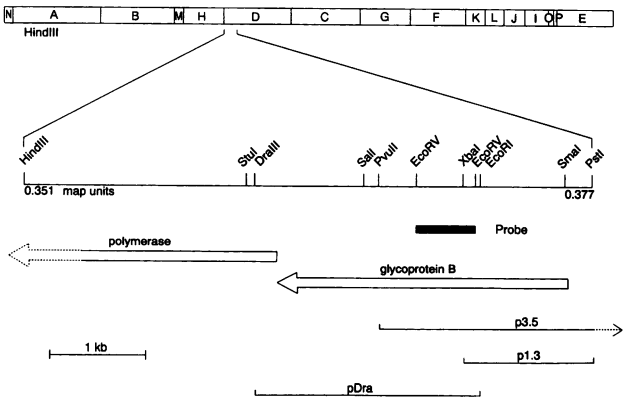

FIG. 1. Localization of the glycoprotein B coding region. Shown are *Hin*dIII cleavage map of the MCMV (Smith) genome (top) and restriction enzymes sites used for gB gene characterization and cloning purposes (middle). The black bar represents the hybridization probe used in the Northern blot experiment. ORFs and plasmids used for cloning purposes are indicated by open arrows and lines, respectively.

by a modified version of the dideoxy sequencing method (42) using *Bal* 31- and exonuclease III-generated subclones of the 3.5-kb *Pvu*II fragment (Fig. 1, p3.5). The 3'-terminal part of the nucleic acid sequence (nt 1400 to 2300) was obtained by the method of Maxam and Gilbert (32) with modifications reported previously (19). Both strands were sequenced, and overlapping clones were used to confirm the sequence. The Genetics Computer Group software package version 7.0 from April 1991 (10) was used for the analysis of the nucleic acid sequence, for the deduction of the amino acid sequence, and for the prediction of the secondary structure of the gB protein. The herpesvirus gB amino acid sequences from the GenBank data base (release 69 from September 1991) were compared.

Northern blot (RNA) analysis and nuclease protection. At different time points postinfection, whole-cell RNA was prepared from MCMV-infected cells and mock-infected control cells following published procedures (7). The RNA was size fractionated by gel electrophoresis, transferred to nitrocellulose filters, and hybridized to a gB-specific, ^{32}P-labeled DNA probe. For the S1 analysis, whole-cell RNA was hybridized to ^{32}P end-labeled DNA fragments and digested with nuclease S1 as described previously (19). Nuclease-resistant fragments were size fractionated on denaturing sequencing gels.

Rapid amplification of cDNA ends. The 3' end of the gB mRNAs was determined according to the protocol of Frohman et al. (14) with the oligonucleotides (dT)17-R1-R0 (5'-AAGGATCCGTCGACATCGATAATACGACTCACTA TAGGGATTTTTTTTTTTTTTTT-3'), R0 (5'-AAGGATC CGTCGACATC-3'), and gB.End (5'-CAGAATTCGTATC TCATCTTCACGAGGC-3'). The primer gB.End corresponds to positions 2650 to 2669 in Fig. 2.

Production of recombinant vaccinia viruses. Vaccinia virus recombinants expressing a truncated (Vac-gB$_{626}$) and the complete (Vac-gB) gB open reading frame (ORF) were generated. For the construction of the recombination plasmid pCS-gB$_{626}$, a 3.5-kb *Pvu*II subclone (Fig. 1, p3.5) of the *Hin*dIII-D fragment (12) constituting two-thirds of the gB ORF and 1.6 kb of upstream sequences was digested with *Sma*I, which cleaves in front of the first ATG, and a *Bam*HI linker was added. Then, the ORF encoding 626 amino acids (aa) of the gB protein was isolated by digestion with *Bam*HI and inserted into the vaccinia virus recombination vector pCS43 (1) downstream of the vaccinia virus early-late promoter p7.5.

Attempts to clone the complete gB ORF into a multicopy plasmid were not successful. Therefore, the gB ORF originally cloned in the low-copy-number plasmid pACYC177 was flanked with the vaccinia virus thymidine kinase sequences and the vaccinia virus p7.5 promoter. In detail, the *Hin*dIII-D fragment cloned into pACYC177 (12) was digested with *Eco*RI and religated, thereby removing nearly the complete *Hin*dIII-D fragment sequences upstream of the gB gene and also part of the gB ORF. Sequences downstream of the gB gene and 600 bp of the vector pACYC177 were removed by digestion with *Dra*III, and religation resulted in the plasmid pDra (Fig. 1). The gB ORF was restored by insertion of a 1.3-kb *Hin*dIII-*Xba*I fragment from a *Pst*I-*Xba*I subclone of the *Hin*dIII-D fragment (Fig. 1, p1.3). This step also removed the remaining *Hin*dIII-D fragment sequences upstream of the gB gene. The resulting plasmid was named pACYC-gB. The sequences constituting the 5' end of the vaccinia virus thymidine kinase gene and the vaccinia virus p7.5 promoter from the plasmid pGS62 (9) were inserted as a 1.2-kb *Hin*dIII-*Sma*I fragment upstream of the gB ORF after digestion of pACYC-gB with *Hin*dIII and *Sma*I. The sequences constituting the 3' end of the vaccinia virus thymidine kinase gene were derived from plasmid pCS43 and inserted as a 1-kb *Hin*dIII-*Sma*I fragment downstream of the gB ORF and the vector sequences. Consequently, the vaccinia virus recombinant Vac-gB contains the gB ORF and 3.5 kb of pACYC177 vector sequences downstream of the p7.5 promoter. The vaccinia virus recombinants were produced by following established procedures (31) using the DNA of vaccinia virus strain Copenhagen and the temperature-sensitive mutant *ts*7 (11).

Preparation of antisera and immunoprecipitation. Antiserum from latently MCMV-infected BALB/c mice was used for the detection of the gB protein in Vac-gB-infected cells. A gB-specific antiserum was prepared by infection of rabbits with the vaccinia virus recombinant Vac-gB$_{626}$. Another gB-specific antiserum used for neutralization assay was prepared by infection of mice with the Vac-gB recombinant. The monospecific rabbit serum was used to identify the glycoprotein B in MCMV-infected cells. Immunoprecipitations were performed as described previously (20). In brief, samples of cell lysates were incubated with 5 μl of antiserum or ascitic fluid. A 50% protein A–Sepharose suspension (Pharmacia) was preincubated with rabbit antibodies specific for mouse immunoglobulin G (Dianova) before addition to the antigen-antibody complexes. The precipitated proteins were separated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis.

MCMV neutralization assay. The neutralizing activity of gB-specific mouse antisera was determined by a plaque reduction assay on mouse embryonal fibroblast monolayers grown in 48-well flat-bottom plates. Serum samples were heated at 56°C for 30 min to inactivate serum complement. A 400-μl volume of the serum dilutions was incubated at 37°C for 30 min with 400 PFU of MCMV contained in 50 μl of medium. Then, 50 μl of rabbit complement was added, and after incubation for another 30 min at 37°C, the plaque assay was carried out.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence reported in this article is M86302.

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tgctctacaa gcatctctcg gtcgactccg agactctcgc cgacgccggt tctcgcgcct cggtcgccga cttatgtcag  204
                SmaI
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GTatgatggt gcgacggggt tAATAAAggt acagtccggc atggaaccca gGATGTTCTa cgccgagaaa gagtacgtgc  3244
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FIG. 2. Nucleotide sequence of the gB gene. The nucleotide sequence from 36 nt upstream of the first 5′ cap site to 54 nt downstream of the second polyadenylation signal of the gB mRNA is shown. The termini of the mRNAs are marked with asterisks. The TATA box sequences, the polyadenylation consensus sequence, and the 3′ end YGTGTTYY consensus sequences are printed in boldface type. The predicted ORF of the mRNA is underlined and set in boldface capital letters. The *Sma*I site at map unit 0.371 is overlined for orientation.

RESULTS

Nucleotide sequence of the glycoprotein B gene. The location of the glycoprotein B (gB) gene is conserved throughout all herpesviruses. A conserved gene block contains the genes for the major DNA-binding protein, a putative transport protein, the glycoprotein B, and the viral DNA polymerase (23). Assuming colinearity between the MCMV genome and the genomes of other herpesviruses, especially HCMV, the putative MCMV gB homolog was expected to be located within the MCMV *Hin*dIII-D fragment upstream of the polymerase gene (Fig. 1) (17). Sequencing of this region revealed a single, long ORF of 2,784 nt (Fig. 2) extending from the ATG codon at positions 250 to 252 to the termination codon at positions 3032 to 3034. The nucleotide sequence of the gene showed 59% homology to the glycoprotein B gene of HCMV.

Expression kinetics of the gB gene. The MCMV replication cycle is divided into three phases: immediate-early, early, and late. Some genes for structural proteins are already expressed in the early phase, other genes are transcribed early and translated in the late phase, and true late genes are only expressed after the onset of the viral DNA replication. To determine to which class the gB gene belongs, RNA from infected cells was prepared at different times after infection, and a Northern blot hybridization was performed. A ^{32}P-labeled 652-nt *Eco*RV fragment (Fig. 1) from the middle of the gB ORF was used to detect the transcript(s) of the gB gene. One major transcript was visible at 24 h postinfection (p.i.) (Fig. 3, lane 6). On the basis of the size of the gB mRNA predicted from the nucleotide sequence, the 3.3-kb transcript is expected to represent the gB mRNA. In addition, two bands of higher molecular masses, which show the same expression kinetics as the 3.3-kb mRNA, were visible. The gB-specific transcript of 3.3 kb appeared at 16 h p.i. (Fig. 3, lane 5) but was not visible at 8 h p.i. The late phase of the MCMV gene expression starts at 16 h p.i. (18). After addition of the DNA polymerase inhibitor phosphonoacetic acid to infected cells, no gB-specific transcript could be detected (Fig. 3, lane 7). Thus, the transcription of the gB gene is restricted to the late phase of infection.

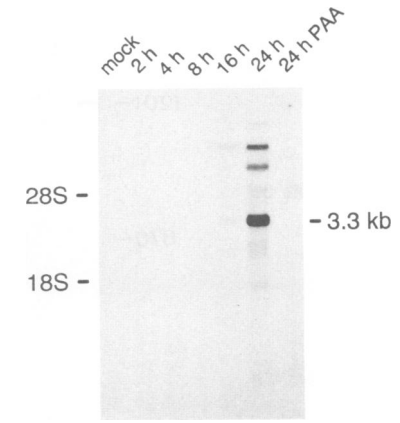

FIG. 3. Transcriptional analysis of gB gene expression kinetics. RNA was prepared at different times postinfection and analyzed by Northern blot hybridization with a gB-specific probe. Lane 1 contained RNA from uninfected cells. Lanes 2 through 6 contained RNA prepared from infected cells at different times p.i. Lanes: 2, 2 h; 3, 4 h; 4, 8 h; 5, 16 h; 6, 24 h. Lane 7 contained RNA isolated from cells infected in the presence of phosphonoacetic acid (PAA) for 24 h.

Identification of the 5′ and 3′ ends of the gB transcript(s). Nuclease S1 analysis was performed to determine the 5′ and 3′ ends of the gB transcript(s). To map the position of the 5′ end of the gB mRNA, a 465-nt fragment was used (Fig. 4A, lane 1). This fragment includes 451 nt of viral sequences extending from an *Hpa*I site (map unit 0.378) to the *Sma*I site (map unit 0.375) (Fig. 1) and 14 nt of vector sequences in front of the *Hpa*I site. The 465-nt fragment was 5′ end labeled at the *Sma*I site. After hybridization of the fragment to RNA isolated from infected cells in the late phase of infection, two nuclease S1-resistant fragments of 135 and 213 nt were visible (Fig. 4A, lanes 3 and 4). This result indicates two different start positions of transcription, 23 and 29 nt downstream of two TATA boxes (Fig. 2, positions −27 to −23 and 47 to 52). The additional protected fragment of 451 nt corresponds exactly to the full length of the viral sequences and results from protection by a transcript of an overlapping gene (33a).

For the location of the 3′ end of the gB mRNAs, a *Sal*I-*Stu*I fragment (map units 0.366 to 0.361; Fig. 1) was used for S1 nuclease analysis (Fig. 4B, lane 1). The 1,201-nt fragment was 3′ end labeled at the *Sal*I site. A protected fragment of approximately 870 nt was found (Fig. 4B, lanes

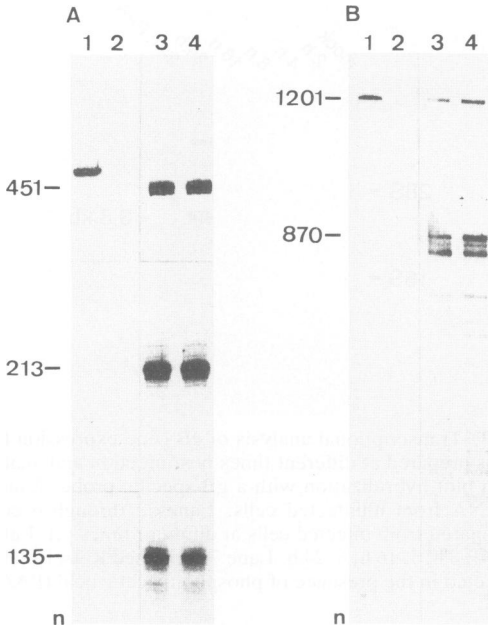

FIG. 4. Structural analysis of the gB gene. The 5′ (A) and 3′ (B) ends of the gB transcripts were determined by nuclease S1 analysis. Lanes 1 show the labeled fragments before the nuclease treatment, and lanes 2 show nuclease-resistant fragments after hybridization to mock RNA. Lanes 3 and 4 show protected fragments after hybridization to RNA of late-phase infected cells at 52 and 58°C, respectively. 5′-end-labeled *Hpa*II cleavage products of pBR322 and the 123-bp and 1-kb ladders (GIBCO BRL) served as size markers. To present all relevant information in one figure, the autoradiographs were cropped and refitted.

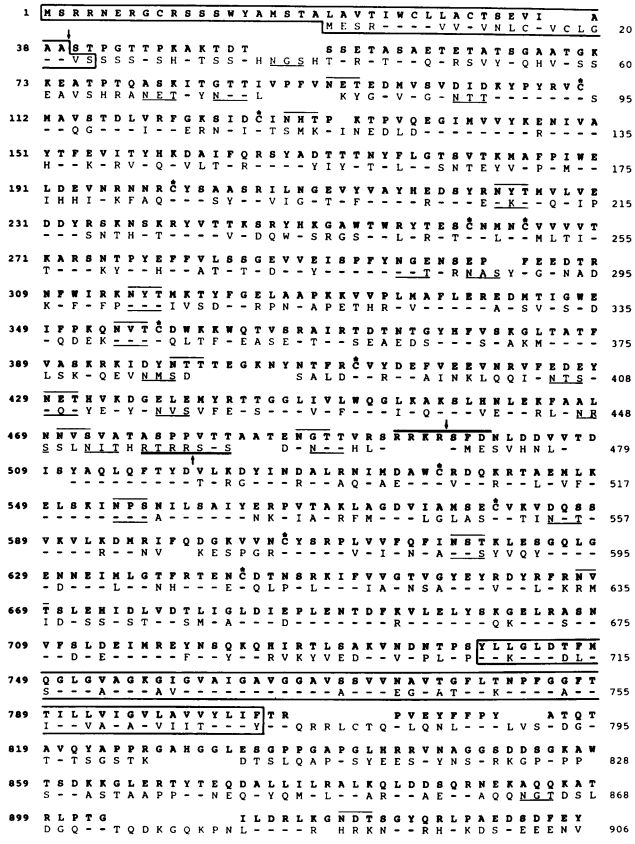

FIG. 5. Comparison of the deduced amino acid sequences of the MCMV (top lines) and HCMV (strain AD169; bottom lines) gB homologs. The sequences are displayed in the one-letter amino acid code. Spaces were inserted for maximal alignment of homologous amino acid sequences. Identical amino acids are marked with dashes in the HCMV sequence, and potential N-linked glycosylation sites are over- or underlined. The putative signal sequence and transmembrane region are boxed. Cysteine residues of the MCMV gB are marked with asterisks. The putative proteolytic cleavage sites are indicated by arrows, and the cleavage signal sequences are underlined twice.

3 and 4), indicating that the two gB transcripts have a common 3′ end. Two putative polyadenylation signals (positions 3127 to 3132 and 3186 to 3191) were found in the nucleotide sequence (Fig. 2) (13). The sequences CGTGT TGT and GATGTTCT (positions 3159 to 3166 and 3216 to 3223), 27 and 25 nt, respectively, downstream of the polyadenylation signals, show homology to the consensus sequence YGTGTTYY, which is frequently found downstream of mRNA 3′ termini (33). The protected fragment of 870 nt locates the 3′ end of the gB mRNAs between the first polyadenylation signal and the first YGTGTTYY signal. The two smaller protected fragments (Fig. 4B, lanes 3 and 4) probably result from overdigestion of the DNA-RNA hybrids due to insufficient hybridization as a consequence of AT-rich sequences around the polyadenylation signal. To determine the 3′ ends of the gB mRNAs more precisely, cDNA was synthesized from the gB mRNAs. The 3′ ends of the cDNAs were amplified according to the protocol of Frohman et al. (14) by using the primers R1 and gB.End. The amplified fragment of approximately 550 nt was cloned into pUC19 and subjected to sequence analysis. According to the sequence data, the 3′ end of the gB mRNAs is located at the C residue at position 3147 (Fig. 2), 15 nt downstream of the first polyadenylation signal. There is no indication that the second polyadenylation signal is also used. Therefore, we conclude that the mRNAs stop predominantly after the first polyadenylation signal.

Primary amino acid sequence and secondary structure analysis of the MCMV glycoprotein B. The amino acid sequence of the MCMV glycoprotein B was deduced from the nucleic acid sequence of the identified ORF (Fig. 5). Hydrophobic and hydrophilic parts of the protein were determined according to the method of Chou and Fasman (8). Several ATGs are located in the 5′ part of the mRNAs. The first two ATGs are almost immediately followed by stop codons and therefore represent minicistrons known from many eukaryotic and viral mRNAs (26). It is not known whether they are used. The following two ATGs are both in frame with the long ORF. Since the third ATG of the mRNA is the first ATG of the long ORF and conforms as well to the Kozak consensus sequence CC(A/G)CC**ATG**C (25) as the second one, translation probably starts here. The gB transcript is translated into a 928-aa protein (Fig. 5), which shows characteristics of a glycoprotein. For the transport into the endoplasmic reticulum, glycoproteins typically possess a signal peptide of 20 to 30 aa. The putative signal peptide of MCMV gB consists of 39 aa with a hydrophobic core from amino acids Ile-25 to Ile-36 (Fig. 5). The signal peptide is probably cleaved after the predicted signal peptidase recognition site Ala-37–Ala-38–Ala-39. In comparison with the HCMV gB, the MCMV gB has a relatively long

signal peptide of 39 aa. However, in comparison with the signal peptides of the pseudorabies virus gB (36) and the bovine herpesvirus type 1 gB (43), which consist of 53 and 67 predominantly hydrophilic aa, the MCMV gB signal peptide is not unusual. The extracellular portion of the protein following the signal peptide consists of 699 aa (Fig. 5, Ser-40–Ser-739). Twelve potential N glycosylation sites are located in this region (Fig. 5, overlined). The Chou-Fasman analysis shows an extended hydrophobic region near the C terminus of the gB, indicating a potential transmembrane domain (data not shown). This putative transmembrane region spans aa 740 to 804 (Fig. 5, boxed). The remaining, basically hydrophilic aa 805 to 928 (Fig. 5) probably form the cytoplasmic tail of the protein.

Comparison of the MCMV gB with the gB homologs of other herpesviruses. To unequivocally demonstrate that the identified gene is the gB homolog of MCMV, the deduced amino acid sequence of the protein (Fig. 5, upper line) was compared with the amino acid sequence of HCMV gB (Fig. 5, bottom line). The gB homologs of the HCMV strains AD169 and Towne are composed of 906 (9) and 907 aa (39), respectively. The MCMV gB and the HCMV gB have 45% identical amino acids that are located mainly in the central portion of the protein, while the N and C termini show little or no identity (Fig. 5). Since all 11 cysteine residues following the signal peptide are conserved (Fig. 5, asterisks), the proteins probably possess similar tertiary structures. Eight of the 12 potential N glycosylation sites of the MCMV gB are found at the same positions as the glycosylation sites of the HCMV gB (Fig. 5, underlined). The gB of HCMV is translated as a polyprotein and subsequently cleaved into two subunits (21, 39). The potential cleavage sites of the HCMV gB proteins are the motifs $RTKR/STD_{463}$ (Towne) (39) and $RTRR/STS_{463}$ (AD-169) (21). A similar motif ($RRKR/SFD_{500}$) was detected in MCMV and probably represents the proteolytic cleavage site.

The amino acid sequence of the MCMV gB protein was also compared by dot matrix analysis with those of the gB homologs of HCMV, Epstein-Barr virus (EBV) and HSV type 1 (HSV-1) (Fig. 6). The MCMV protein is similar to all other gB homologs, the transmembrane region being more conserved than other parts of the protein. The highest degree of homology exists between the gBs of MCMV and HCMV, which have 45% of their amino acids in common. This was expected, since the two betaherpesviruses are more related to each other than to the alphaherpesviruses (HSV-1) and gammaherpesviruses (EBV). The gB homologs of MCMV and EBV and MCMV and HSV-1 show 34 and 26% identity, respectively.

Identification of the MCMV glycoprotein B in infected cells. To identify the gB gene products in infected cells, immunoprecipitations were performed. Proteins of MCMV-infected cells were labeled with [^{35}S]methionine from 16 to 28 h p.i. Proteins were precipitated from cell lysates by a monospecific serum produced in a rabbit against the vaccinia virus recombinant $Vac\text{-}gB_{626}$. The autoradiography of the gel is shown in Fig. 7A. The gB-specific serum precipitated polypeptides with apparent molecular masses of 130, 105, and 52 kDa from lysates of MCMV-infected cells but not from lysates of noninfected cells (Fig. 7A, lanes 3 and 4). The sizes of the proteins are similar to those reported for the HCMV gB.

Expression of the MCMV gB ORF by a recombinant vaccinia virus. Recombinant vaccinia viruses expressing the MCMV gB ORF were constructed as described in Materials and Methods. The acquisition of the MCMV DNA by the

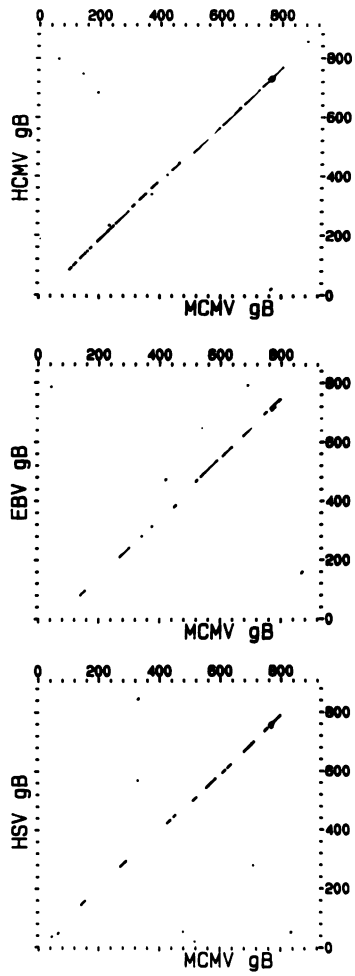

FIG. 6. Dot matrix comparison of the MCMV gB homolog and the gB homologs of HCMV, EBV, and HSV-1. The following parameters were used: window, 15 aa; and stringency, 12 aa. The N termini of the sequences are in the bottom left-hand corner in each graph.

recombinant vaccinia virus Vac-gB was verified by hybridization with a gB-specific DNA probe (data not shown). The gB gene products synthesized in Vac-gB-infected CV-1 cells were characterized by immunoprecipitation of [^{35}S]methionine-labeled cell proteins with a polyvalent anti-MCMV serum. In Vac-gB-infected cells, a protein with the apparent molecular mass of 130 kDa was found (Fig. 7B, lane 4), which comigrated with the MCMV gB precursor (compare Fig. 7A, lane 4 and Fig. 7B, lane 4).

Identity of the MCMV glycoprotein B with the MCMV major envelope glycoprotein. The major envelope glycoprotein of MCMV has been described by Loh et al. (27, 28). Three proteins, gp 150, gp 52, and gp105, which were derived from a common precursor of 128 kDa were identified on the surface of the virion (27). This closely resembles the situation described for the HCMV gB gene products. To test whether the proteins of the major envelope glycoprotein complex are the translation products of the identified gB gene, the protein expressed by the recombinant vaccinia virus Vac-gB was tested with the monoclonal antibody 2E8.21A against the major envelope glycoprotein (28). The monoclonal antibody precipitated proteins of 130, 105, and

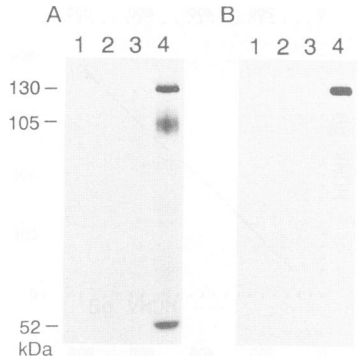

FIG. 7. Detection of gB in MCMV- and Vac-gB-infected cells. Proteins were labeled at 16 (MCMV) or 12 (Vac-gB) h p.i. for 12 h. (A) Proteins of lysates from uninfected (lanes 1 and 3) or MCMV-infected (lanes 2 and 4) cells were precipitated with preimmune serum (lanes 1 and 2) or the gB-specific serum (lanes 3 and 4). (B) Proteins of lysates from CV-1 cells infected with wild-type vaccinia virus (lanes 1 and 3) or Vac-gB (lanes 2 and 4) were precipitated with preimmune serum (lanes 1 and 2) or anti-MCMV serum (lanes 3 and 4). The molecular masses of the gB polypeptides are shown on the left.

52 kDa from lysates of MCMV-infected cells (Fig. 8, lane 2). Proteins of the same size were precipitated with the gB-specific antiserum (Fig. 7A, lane 4). In Vac-gB-infected cells, a protein which has the same molecular mass as that of the gB precursor form was detected (Fig. 8, lane 4). Thus, the glycoprotein B and the major envelope glycoprotein are identical.

Induction of neutralizing antibodies by the Vac-gB recombinant. A gB-specific antiserum was raised in mice by infection with the Vac-gB recombinant and tested for neutralizing activity in a plaque reduction assay. A mouse preimmune serum and an anti-MCMV serum raised in mice by infection with MCMV were included in the plaque reduction assay for comparison. Whereas the mouse preimmune serum was not able to neutralize the virus, the anti-gB and anti-MCMV antisera showed considerable neutralizing activity (Table 1). The anti-gB serum neutralized MCMV in the presence of complement. The anti-MCMV serum showed a higher level of neutralizing activity and was also capable of neutralizing the virus in the absence of complement, although the complement-independent neutralizing activity was weak.

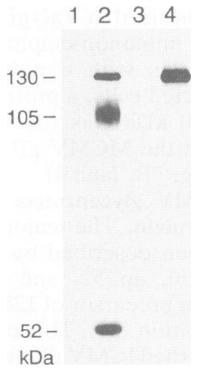

FIG. 8. Identity of the MCMV glycoprotein B with the MCMV major envelope glycoprotein. Polypeptides in lysates from uninfected (lane 1), MCMV-infected (lane 2), wild-type vaccinia virus (strain Copenhagen)-infected (lane 3), and Vac-gB-infected (lane 4) cells were precipitated with the monoclonal antibody 2E8.21A. The molecular masses of the gB polypeptides are indicated on the left.

TABLE 1. Neutralization of MCMV by different antisera

Antiserum	MCMV plaque reduction (%)[a]	
	With complement	Without complement
Preimmune	0 (1:10)	0 (1:10)
Anti-gB	73 (1:200) 41 (1:400)	0 (1:10)
Anti-MCMV	65 (1:1,600) 36 (1:3,200)	70 (1:20) 36 (1:40)

[a] Serum dilutions are indicated in parentheses. For neutralizing sera, dilutions that give more than 50% and less than 50% plaque reduction are shown.

DISCUSSION

The MCMV glycoprotein B gene was identified, sequenced, and expressed by recombinant vaccinia virus. The gene product in Vac-gB-infected cells showed the same migration property as the gB precursor in MCMV-infected cells. Although the mature gB subunits were not found in Vac-gB-infected cells, mice immunized with the recombinant developed antibodies that neutralized MCMV in vitro.

The identification of the MCMV gB gene was possible because the gene is located in a conserved block of genes (23). The genes for glycoprotein B, a putative transport protein, and the major DNA-binding protein are organized in exactly the same order in HCMV, EBV, and HSV-1. The viral DNA polymerase gene is located downstream of the gB gene in HCMV and EBV but not in HSV-1 (23). Since MCMV is more related to HCMV than to other herpesviruses, the MCMV gB gene was expected to be located upstream of the polymerase gene. Sequencing of this region revealed an ORF of 2,784 nt.

The assumption that the identified ORF represents the gB gene was further strengthened by the high degree of homology between the deduced amino acid sequence of the putative MCMV gB homolog and the amino acid sequences of the gB homologs of HCMV, HSV-1, and EBV. The highest degree of homology was found between the gBs of the two members of the betaherpesviruses, MCMV and HCMV. Considerable homology to gBs of other herpesviruses, e.g., alphaherpesviruses (HSV-1) and gammaherpesviruses (EBV), was also found. The recently published sequence of the carboxy terminus of the MCMV gB of strain K181 is almost identical to our sequence, with the exception of some amino acid exchanges and a small deletion of 7 aa at position 805 (13). These data indicate that the function of the gB allows only subtle differences at certain positions. The deletion might point to a less important region of the protein.

Comparisons of the amino acid sequences of all known gB homologs show that a significant portion of the amino acid sequence is conserved among all herpesviruses (9, 22). It has also been reported previously that 10 cysteine residues are perfectly aligned in all herpesvirus gB homologs (22). Therefore, the tertiary structure of the proteins appears to be conserved too. This notion is confirmed by the fact that the gB homolog from one herpesvirus can functionally comple-

ment the gB deficiency of another closely related virus (35). Thus, the glycoprotein B, an essential protein, is highly conserved throughout all herpesviruses not only with regard to genome location, primary amino acid sequence, and secondary and tertiary structure but also in its function.

The investigation of the expression kinetics of the MCMV gB gene revealed a difference from that of HCMV. Whereas Spaete et al. report the appearance of the gB mRNA of the HCMV strain Towne as soon as 4 h p.i. and well in advance of gB protein synthesis, the MCMV gB mRNA is not visible until late in infection (39). Transcription of the MCMV gB gene is blocked by the DNA replication inhibitor phosphonoacetic acid. Thus, the MCMV gB gene is a true late gene and there is no evidence for posttranscriptional regulation such as that suggested for the HCMV gB gene (strain Towne) (39). However, despite the different transcriptional patterns, the expression of the protein is restricted to the late phase in both viruses. In addition to the expected 3.3-kb gB-specific transcript, two RNAs of higher molecular masses are visible. The existence of additional, longer transcripts has also been reported for the HCMV gB gene (39) and the HCMV and MCMV polymerase genes (13, 24). It has been proposed that these HCMV transcripts might represent read-through products of the gB transcript which would normally terminate at the polyadenylation signal within the DNA polymerase-coding region (24). Whether a similar situation exists for the larger MCMV transcripts remains to be established.

To conduct in vivo protection experiments, the gB ORF was engineered into vaccinia virus. At first, it was possible to clone only part of the gB gene into a vaccinia virus recombination plasmid. For unknown reasons, the whole gB ORF could not be inserted into a commonly used vaccinia virus recombination plasmid. Similar problems have been reported elsewhere for the cloning of the HSV gB gene (5) and the HCMV gB gene (29). These problems could be due to a gene product which is toxic for the bacteria. Therefore, instead of subcloning the gene, it was flanked by the vaccinia virus sequences from recombination vectors pGS62 and pCS43. The resulting recombination vector was based on the low-copy-number plasmid pACYC177. Because of a copy number of only 20 plasmids per bacterial chromosome (6), the bacteria are probably able to tolerate small amounts of the putative toxic gene product.

The protein expressed by the recombinant vaccinia virus Vac-gB has the same size (130 kDa) as the gB precursor protein in MCMV-infected cells. However, the subunits of 105 and 52 kDa, which are visible in MCMV-infected cells, could not be detected in Vac-gB-infected cells. For the HCMV gB, it has been proposed that a cellular protease cleaves the protein (38), which probably belongs to the subtilisinlike serine protease family (2). The putative cleavage site of the MCMV gB corresponds to the consensus cleavage motif of these proteases. For vaccinia virus-expressed substrates, it has been shown that cleavage by this serine protease family does not occur late in infection (3). In fact, when Vac-gB-infected cells were labeled from 3.5 to 6.5 h after infection, faint traces of the processed gB subunits could be detected (data not shown).

Immunization with the Vac-gB recombinant induced a considerable level of neutralizing activity. However, the neutralizing activity level of the anti-MCMV serum was higher. It has been reported elsewhere that a significant portion of the human humoral immune response against HCMV is directed against the HCMV gB (4). Further experiments will clarify whether the higher neutralizing activity of the anti-MCMV serum is due to a titer of anti-gB antibodies higher than that of the Vac-gB serum or whether the anti-MCMV serum also contains neutralizing antibodies against other MCMV proteins.

In MCMV-infected cells, three proteins with the apparent molecular masses of 130, 105, and 52 kDa were detected with an anti-gB serum. Thus, the products of the MCMV gB gene have approximately the same sizes as their HCMV counterparts (4, 9, 39). In addition, the proteins have exactly the same sizes as the proteins of the major envelope glycoprotein complex of MCMV (27, 28). The reactivity of the monoclonal antibodies 2E8.21A and 8G5.21A (28) with the Vac-gB gene product in Western blotting (data not shown) and immunoprecipitations confirmed the identity between the gB gene products and the major envelope glycoprotein complex.

As outlined above, the herpesvirus gB homologs are extremely conserved in several aspects. Thus, the MCMV gB is suitable for studying the immune response against glycoprotein B in a natural virus-host model. The Vac-gB construct will enable us to study the protective potential of this protein. This may be of interest in view of the use of the glycoprotein B as a subunit vaccine.

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